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EPINEPHRINE AND DOPAMINE CONTENT OF LARVAE AND SPAT OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

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Abstract

Oyster larvae and spat of varying ages were homogenized and analyzed for catecholamine content using high-performance liquid chromatography with electrochemical detection. Norepinephrine (NE) and dopamine (DA) were found in appreciable quantities, with more DA present than NE. The presence of epinephrine was equivocal. Norepinephrine levels were low in young larvae (0.062 ± 0.035 pg/μg protein), then increased to an intermediate level (0.517 ± 0.031 pg/μg protein) for most of the larval period and finally increased again just before metamorphosis to 1.08 ± 0.09 pg/μg protein. Dopamine levels in the corresponding larval groups did not change significantly, with 11.5 ± 1.9, 8.58 ± 0.98, and 7.47 ± 0.92 pg DA/μg protein, respectively. The levels of NE (1.00 ± 0.04 pg/μg protein) and DA (7.13 ± 0.29 pg/μg protein) present in spat following metamorphosis were not significantly different from the pre-metamorphic values. The presence of NE in oyster larvae at the time of metamorphosis supports the hypothesis of a role for endogenous NE in mediating metamorphosis.

Introduction

Although norepinephrine (NE) is a major neurotransmitter in the vertebrates, its role(s) in the invertebrates has not been established (Welsh, 1972; Leake and Walker, 1980; Gospe, 1983). Although low levels of NE have been found in most invertebrate phyla, there has been little evidence of a functional role for NE or for the presence of receptors that might mediate such a function. However, Coon *et al.* (1985) have demonstrated that larvae of the Pacific oyster *Crassostrea gigas* (Thunberg), can be induced to metamorphose from a pelagic larval stage to a sessile juvenile stage by a brief (<1 h) exposure to NE or epinephrine (EPI), and subsequent research (see below) suggests catecholamines may be directly involved in natural oyster metamorphosis.

Prior to metamorphosis, *C. gigas* larvae, like many marine invertebrate larvae, are pelagic and must spend some period of growth and development in the plankton. At a certain developmental stage, the larvae become competent to respond to appropriate environmental stimuli, which can cause them to proceed through a series of complex stereotyped behaviors called settlement. If a suitable substrate is encountered during settlement, the larvae will attach to it irreversibly. After attachment, the larvae undergo a morphological and physiological metamorphosis to a sessile juvenile stage. Norepinephrine- and EPI-induced metamorphosis is independent of settlement and attachment; upon exposure to NE or EPI, the larvae sink to the bottom and metamorphose without attaching (Coon *et al.*, 1985).

Coon and Bonar (submitted) have shown that induction of metamorphosis by

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EPI is mediated by receptors pharmacologically identified as alpha$_1$-adrenoceptors, thus suggesting that endogenous NE or EPI may play some role in the metamorphosis of *C. gigas*. Other natural adrenergic agonists, such as dopamine (DA) and octopamine (OCT), for which there is growing evidence for neurotransmitter functions in the molluscs (Gospe, 1983; David and Coulon, 1985), are ineffective inducers of metamorphosis.

We have found no reports in the literature of levels of endogenous catecholamines in molluscan larvae. Norepinephrine has been found in the nervous systems and other tissues of adults of some molluscan species (Juorio and Killick, 1972; Guthrie *et al.*, 1975; Burrell and Stefano, 1983) but except for a report on *Helix* by Osborne (1984), EPI has not been found. Dopamine is considered to be the major catecholamine in the molluscs (Welsh, 1972; Gospe, 1983). This study was undertaken to determine the levels of endogenous catecholamines (NE, EPI, and DA) in *C. gigas* larvae.

Materials and Methods

Materials

Oyster larvae were obtained from the Coast Oyster Hatchery (Quilcene, Washington) as previously described (Coon *et al.*, 1985) and homogenized upon arrival. Different size classes of larvae were shipped separately and prior to homogenization were further sorted using Nitex screens of appropriate mesh sizes. The mean size of each group of larvae homogenized was determined by measuring the anterior-posterior length of 25 individuals. All references to larval age are days after fertilization and refer to the age of the larvae when they were shipped from the hatchery.

Post-metamorphic juveniles (called spat) used in this study were produced in our laboratory by allowing batches of larvae to settle and attach onto a clean glass surface for two hours, following which they were carefully scraped free with a razor blade. The spat were then carefully culled and those with damaged shells were discarded. Undamaged spat completed metamorphosis normally and were homogenized at 6 or 24 hours after the beginning of the two hour settlement period.

All chemicals used were the highest grade available from Sigma Chemical Co. (St. Louis, Missouri). The Coomassie Blue dye reagent was obtained from BioRad (Richmond, California). Ultra-pure water (distilled, deionized, and reverse osmosis-purified: DDW) was used for all analyses.

Tissue preparation

Prior to homogenization, larvae and spat were washed twice with iced 0.2 μm filtered seawater. After washing, a quantity of whole larvae or spat was homogenized in a glass tissue grinder in 1.0 ml iced 0.4 *N* perchloric acid (PCA) containing 4 m*M* reduced glutathione, 5 m*M* EGTA, and 7.2 n*M* dihydroxybenzylamine (DHBA) as an internal standard. Homogenates were stored at −70°C until final processing for analysis. Just prior to analysis, homogenates were thawed and centrifuged at 15,000 $\times g$ for 5 minutes and the catecholamines were extracted from the supernatant using alumina (Anton and Sayre, 1962). An 800 μl aliquot of the supernatant was diluted to 3 ml with DDW followed by the addition of 2.0 ml of 1.5 *M* Tris (pH = 8.6) containing 55 m*M* Na_2 EDTA. Approximately 50 mg of acid washed alumina was added and each tube was agitated for 15 minutes by gentle inversion on a vertical turntable. The alumina was washed twice with DDW before transferring it to a Microfilter assembly (Bioanalytical Systems, Inc., West Lafayette, Indiana: BAS). The

remaining wash was removed by centrifugation and the catecholamines were eluted from the alumina with 100 μl of 0.1 *N* PCA. This extract was injected directly into the HPLC system.

Catecholamine analysis

Catecholamines were separated using a 250 × 4.6 mm I.D. column with 5 μm, C-18 reverse phase packing (Biophase RP-18, BAS). The mobile phase contained 95 m*M* monochloroacetic acid, 1.3 m*M* Na_2EDTA and 0.91 *M* acetonitrile and was adjusted to pH = 3.00–3.05 with NaOH (85 m*M* final concentration). Sodium octyl sulfate (1.2 m*M* final concentration) was added as an ion pairing agent (Knox and Jurand, 1976; Krstolovic, 1982). The flow rate was adjusted to 1.5 ml/min.

Detection of catecholamines was accomplished using an LC-4B amperometric detector with a glassy carbon electrode (BAS) set at an oxidizing potential of 750 mV *versus* a Ag/AgCl reference electrode (Riggin and Kissinger, 1977; Krstolovic, 1982). The detector was set at 0.5 nA/V for all catecholamines except DA, which, because of its high concentration, required a detector setting of 5.0 nA/V; the recorder was set at 1 V full scale.

Catecholamines were identified by comparison of their retention times to those of known standards as a function of increasing concentrations of acetonitrile (0.91–1.7 *M*) and by comparison of their peak heights to known standards as a function of increasing electrode potentials (600–900 mV). Catecholamines were quantified using peak heights compared to a standard curve which was calculated at the beginning of each day. Catecholamine concentrations were expressed as pg of free base/μg protein, with protein content determined by the Coomassie Blue dye binding method (Bradford, 1976) using bovine serum albumin as the standard. Overall recovery for the procedure was about 60% and data were corrected for recovery efficiency. Data were analyzed at the 95% confidence level using analysis of variance and the Newman-Keuls method to test for differences between means (Snedecor and Cochran, 1967).

Results

Figure 1 shows typical chromatographs obtained for a solution of catecholamine standards (A) and for alumina extracts of homogenized competent oyster larvae (B). It can be seen that NE, DA, and the internal standard, DHBA, were well separated from all other peaks and that there were few extraneous peaks. Peak I (probably dihydroxyphenylalanine), peak II (probably epinephrine), and peak III (unknown) in the chromatograph of the larvae could not be adequately identified or quantified so were not included in this report, however these peaks are the subjects of continuing research. Dopamine was the dominant catecholamine in all larval samples. Note the change in scale used for the DA peak in Figure 1(B).

Figure 2 shows the amount of NE present in oyster larvae and metamorphosed spat as a function of shell length. These data are summarized in Table I. Norepinephrine is at a low level (0.062 ± 0.035 pg/μg protein) in young larvae up to about 120 μm in length (5–6 days old). There is an increase in NE content to (0.517 ± 0.031 pg/μg protein) by about 155 μm length (8–9 days old). Norepinephrine remains at about this level for most of the remainder of the larval period until another increase to 1.08 ± 0.09 pg/μg protein occurs at around 300 μm length (18–19 days old). This increase corresponds closely with the onset of metamorphic competence. Figure 2 also shows that during the first 24 h following metamorphosis, the level of NE (1.00 ± 0.04 pg/μg protein) does not change from pre-metamorphic levels.

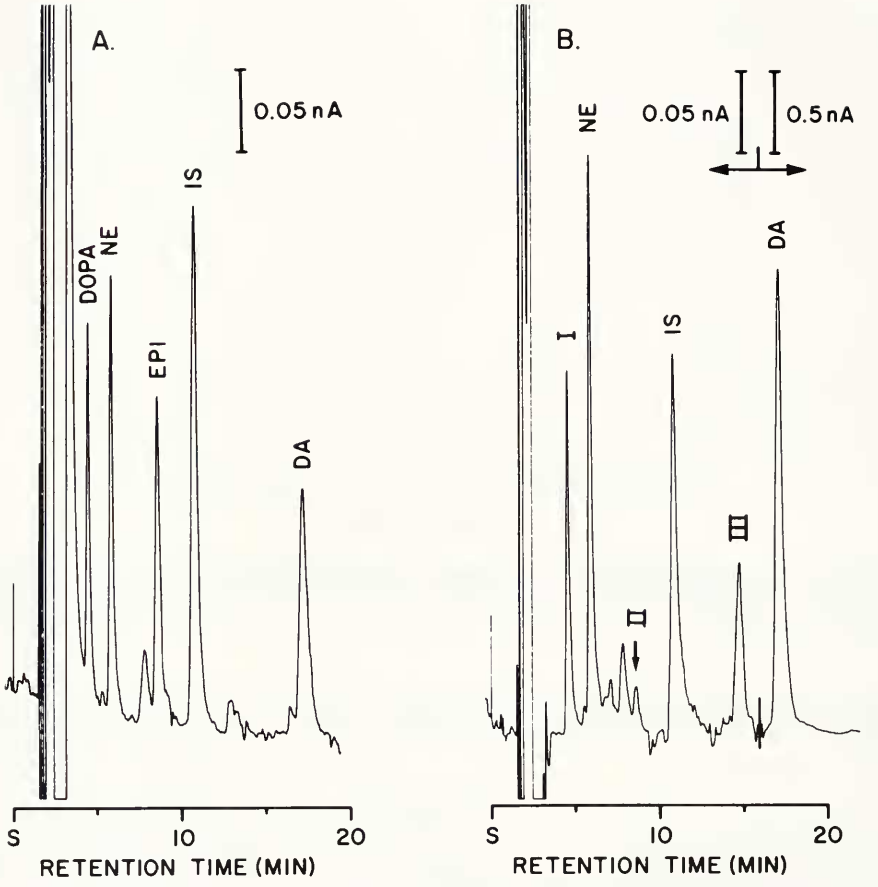

FIGURE 1. Typical chromatographs obtained using HPLC with electrochemical detection as described in the text. (A) Solution of catecholamine standards (DOPA: 230 pg; NE: 340 pg; EPI: 340 pg; IS: 460 pg; DA: 340 pg). (B) Alumina extract of a homogenate of whole, competent oyster larvae. Note the change in scale for the DA peak. DOPA: dihydroxyphenylalanine; NE: norepinephrine; EPI: epinephrine; IS: internal standard, dihydroxybenzylamine; DA: dopamine. Unidentified peaks: I (probably dihydroxyphenylalanine), II (probably epinephrine), III (unknown).

Figure 3 shows the corresponding amounts of DA present in the larvae and spat as a function of larval size. These data are also shown in Table I along with the NE data. It can be seen that there is significantly more DA than NE present in the larvae and spat. There are no statistically significant trends in the DA data although there is a tendency for higher values in smaller larvae; the DA content per μg protein is relatively constant throughout the larval life. The DA levels in small, middle-sized, and large larvae are 11.5 ± 1.9, 8.58 ± 0.98, and 7.47 ± 0.92 pg/μg protein, respectively. Like NE, the DA levels (7.13 ± 0.29 pg/μg protein) do not change during the first 24 h following metamorphosis compared to pre-metamorphic values. Unlike the NE content, there is no increase in the DA content corresponding to the onset of larval competence. The ratio of DA:NE decreases during larval development from a value of about 180 for small larvae to about 17 for middle-sized larvae and finally to about 7 for the largest (competent) larvae and spat. Because there is little change in DA

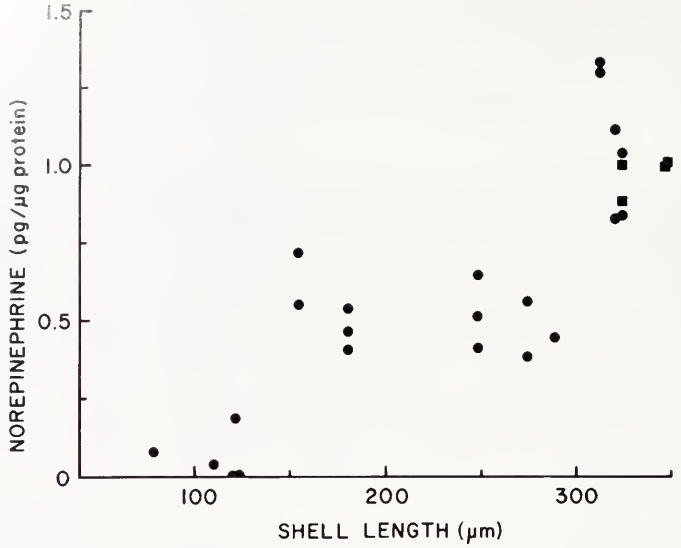

FIGURE 2. Norepinephrine content of oyster larvae and spat as a function of anterior-posterior length. Each point represents a single determination. Circles: larvae; squares: spat 6 and 24 hours after the onset of metamorphosis.

levels during this time, most of the decrease in the DA:NE ratio is due to increases in NE.

DISCUSSION

The present study extends knowledge of the occurrence of catecholamines in molluscs to the larval stages. Norepinephrine and DA are present in appreciable amounts in *C. gigas* while the presence of EPI is still equivocal. However, it is difficult to compare directly the absolute concentrations of catecholamines reported here, which are determined from whole animal extracts (including the proteinaceous shell matrix), with those found in the literature pertaining to adults, which are usually deter-

TABLE I

Catecholamine content of oyster larvae and spat

Shell length (μm)	Norepinephrine content (pg/μg protein)	Dopamine content (pg/μg protein)	DA:NE ratio
Larvae			
[illegible]-122	0.062 ± 0.035 (a)[1]	11.5 ± 1.9 (d)	180
[illegible]-288	0.517 ± 0.031 (b)[2]	8.58 ± 0.98 (d)	17
[illegible]-324	1.08 ± 0.09 (c)	7.47 ± 0.92 (d)	6.9
Spat			
324-[illegible]	1.00 ± 0.04 (c)	7.13 ± 0.29 (d)	7.1

[1] All values are the mean ± S.E.M.
[2] Means followed by the same letter in parentheses are not significantly different from one another.

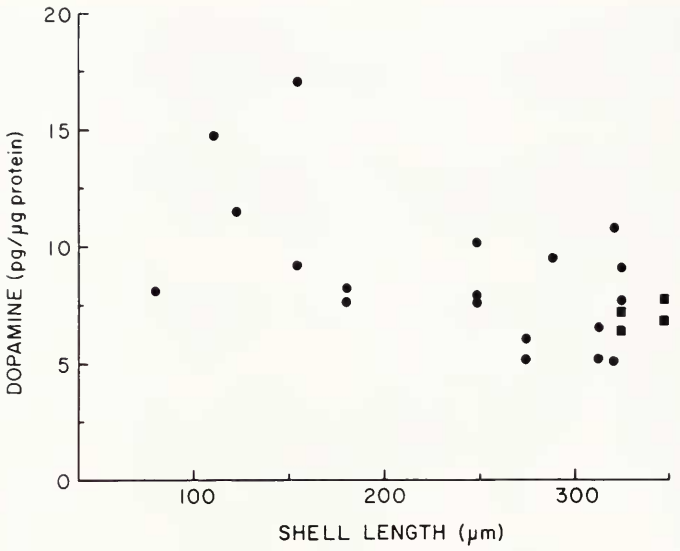

FIGURE 3. Dopamine content of oyster larvae and spat as a function of anterior-posterior length. Each point represents a single determination. Circles: larvae; squares: spat 6 and 24 hours after the onset of metamorphosis.

mined from isolated tissues. Nonetheless, the DA:NE ratio may provide a point of comparison although such comparisons must be made cautiously because of differences in the tissues studied.

As shown in Table I, the DA:NE ratios were 180, 17, and 6.9 for small, middle-sized, and large (competent) larvae, respectively; the ratio for new spat was 7.1, similar to the value for competent larvae. These values agree generally with values from the literature. For example, the DA:NE ratio in molluscan neural tissue was found to range from about 11–13 in *Mytilus* (Burrell and Stefano, 1983) to 20–85 in *Helix* (Guthrie *et al.,* 1975; Osborne, 1984), and with ratios of 17 and 6 for *Helix* kidney and ventricle, respectively (Guthrie *et al.,* 1975). Thus it can be seen that in small oyster larvae the DA:NE ratio is somewhat higher than the literature values, in middle-sized larvae the ratio is similar to literature values and for competent larvae and spat the ratio is somewhat lower.

The presence of NE in competent oyster larvae, along with our previous reports that NE will induce metamorphosis (Coon *et al.,* 1985) and that induction is mediated by putative alpha$_1$-adrenoceptors (Coon and Bonar, submitted), suggests that endogenous NE has a functional role in oyster metamorphosis. This is believed to be the first evidence for a specific functional role for NE in the molluscs. Glaizner (1968) has reported an identified neuron in the snail *Helix* which is stimulated specifically by NE, however the function of this neuron is unknown.

The apparent increase in NE levels near the onset of metamorphic competency is intriguing, especially in light of its presumed involvement in metamorphosis. Since little is known about the physiological changes that accompany competency, the role of NE in this phenomenon is difficult to evaluate. Perhaps noradrenergic neurons are forming, or existing neurons are accumulating NE, either in preparation for involvement in the processes of settlement and/or metamorphosis, or for post-metamorphic functions. The observation that NE levels do not change following metamorphosis

implies that NE has some additional function(s) besides its suspected role in metamorphosis.

Although peak II (see Fig. 1B) was present in nearly all samples tested, its identity as EPI could not be unequivocally verified. Its magnitude was found to vary greatly, independent of larval size and several sample preparation procedures. Although peak II always coeluted with authentic EPI as a function of changing concentrations of acetonitrile in the mobile phase, in some samples its peak height did not correlate well with authentic EPI as a function of the detector potential, indicating the presence of a contaminating compound in some samples. We have not yet been able to resolve this problem, but in light of the fact that EPI is the most effective catecholaminergic inducer of metamorphosis (Coon *et al.,* 1985; Coon and Bonar, submitted) and that there is only one other report of EPI in a mollusc (Osborne, 1984), we are continuing to pursue the identification of peak II.

The process of oyster metamorphosis is complex, involving many different tissues and including histolysis, histogenesis, and the accompanying biochemical and physiological changes (Cole, 1938; Hickman and Gruffydd, 1971). The control mechanisms coordinating these diverse changes are unknown (Burke, 1983; Chia and Rice, 1978; Hadfield, 1978) and the role of NE in this process is likewise unclear. Norepinephrine could be acting as a neurotransmitter within the central nervous system to release a hormone that would act on the peripheral target tissues (centralized receptor theory), analogous to the control of neurosecretory release of a growth hormone by dopamine in the snail *Lymnaea stagnalis* (Stoof *et al.,* 1984). Alternatively, NE could be acting directly on the target tissues (peripheral receptor theory) either as a neurotransmitter or as a hormone. An action on target tissues is suggested in another mollusc, the nudibranch *Phestilla sibogae,* where NE at high concentrations induces low levels of partial metamorphosis in which a specific larval tissue, the velum, is lost but subsequent morphological changes do not occur (Hadfield, 1984). The present data give no indication of the location of either NE or DA within the larvae because whole animal homogenates were used. Further research on the localization of NE and the alpha$_1$-adrenoceptors is required to more fully evaluate the actual role of NE in oyster metamorphosis.

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